Development 137, 551-559 (2010) doi:10.1242/dev.042424

BMP signaling induces digit regeneration in neonatal mice

Ling Yu[1,*], Manjong Han[1,*], Mingquan Yan[1], Eun-Chee Lee[1], Jangwoo Lee[1] and Ken Muneoka[1,2,†]

SUMMARY

The regenerating digit tip of mice is a novel epimorphic response in mammals that is similar to fingertip regeneration in humans. Both display restricted regenerative capabilities that are amputation-level dependent. Using this endogenous regeneration model in neonatal mice, we have found that noggin treatment inhibits regeneration, thus suggesting a bone morphogenetic protein (BMP) requirement. Using non-regenerating amputation wounds, we show that BMP7 or BMP2 can induce a regenerative response. BMP-induced regeneration involves the formation of a mammalian digit blastema. Unlike the endogenous regeneration response that involves redifferentiation by direct ossification (evolved regeneration), the BMP-induced response involves endochondral ossification (redevelopment). Our evidence suggests that BMP treatment triggers a reprogramming event that re-initiates digit tip development at the amputation wound. These studies demonstrate for the first time that the postnatal mammalian digit has latent regenerative capabilities that can be induced by growth factor treatment.

KEY WORDS: BMP7, BMP2, Digit, Regeneration, Blastema, Endochondral ossification, Mouse

INTRODUCTION

A major goal of regeneration studies is to design practical approaches to stimulate a clinically relevant regenerative response. The strategies for regenerative medicine are many and include the bioengineering of tissues and organs for implantation as replacement parts, the introduction of artificial or biological scaffolds into an injury site to facilitate a regenerative response and the development of stem cells that have the potential for use in cell-based therapeutic applications (Muneoka et al., 2008). An alternative approach, which has a long history in experimental biology, is based on the idea that by understanding endogenous regenerative capabilities we can develop strategies to enhance regenerative ability and thus overcome regenerative failure. Most of the work in this arena has focused on the exceptional regenerative potential of the urodele amphibian limb, which undergoes a complex epimorphic response involving blastema formation, pattern formation and morphogenesis and redifferentiation (see Carlson, 2007; Stocum, 2006). To study regenerative failure in amphibians, the anuran tadpole limb that transitions from a regeneration-competent stage to a regeneration-incompetent stage has proved a useful model to identify specific barriers of a regeneration response (see Yakushiji et al., 2009). A similar approach in higher vertebrates involves investigating amputation injury responses in embryonic limbs to identify factors required for regeneration (Muller et al., 1999; Muneoka and Sassoon, 1992). This strategy has been successful in inducing chick limb bud regeneration by treatment with either fibroblast growth factor 2 (FGF2) or FGF4 (Kostakopoulou et al., 1996; Taylor et al., 1994), and recent studies suggest that the WNT signaling pathway might also play a key role (Kawakami et al., 2006). These studies suggest that the non-regenerating amputation wound in amphibians and birds display regenerative potential and that treatment with key growth factors can effectively transition a non-regenerative healing response to a regeneration response.

[1]Division of Developmental Biology, Department of Cell and Molecular Biology and [2]Center for Bioenvironmental Research, Tulane University, New Orleans, LA 70118, USA.

*These authors contributed equally to this work
†Author for correspondence (kmuneoka@tulane.edu)

Accepted 8 December 2009

In mammalian limbs there are few instances in which a regenerative response has been induced experimentally. Following the lead of Marcus Singer's studies on the neurotrophic influence in amphibian limb regeneration (Singer, 1952), Mizell reported on induced regeneration of amputated newborn opossum hind limbs by implantation of neural tissues (Mizell, 1968). However, in an effort to replicate these studies, Fleming and Tassava reported that anatomical variability in the timing of skeletal outgrowth of the opossum hindlimb, and not neural tissue grafts, could account for the partial regenerative response reported by Mizell (Fleming and Tassava, 1981; Mizell, 1968). The developing mammalian limb bud possesses an endogenous ability to undergo a partial regenerative response in vitro and in vivo, indicating that regenerative ability is enhanced during development (Chan et al., 1991; Deuchar, 1976; Wanek et al., 1989). In utero amputation of developing digits in the mouse results in a level-dependent regenerative response that correlates with the distal expression domain of the homeobox-containing gene *Msx1* (Reginelli et al., 1995). Han et al. (Han et al., 2003) found that *Msx1* mutant mice displayed a regeneration defect that can be rescued by treatment with exogenous BMP4, and that treatment of wild-type digit amputations with the BMP antagonist noggin inhibited regeneration. These studies identified the BMP signaling pathway as necessary for a regenerative response in the embryonic digit.

In postnatal humans, the only part of the body that has the capacity to regenerate is the fingertip. This regenerative response was first documented in children and later reports indicate a similar response in adults (Muneoka et al., 2008). Fingertip regeneration is amputation-level-dependent: distal amputations successfully regenerate, whereas proximal level amputations fail (Han et al., 2008). The digit tip of the mouse responds similarly to amputation and represents a model for human regeneration. Indeed, amputated human fetal digits initiate a regenerative response in culture that is similar to the mouse in that the response is associated with the expression of MSX1 (Allan et al., 2006). Digit tip regeneration in mice readily occurs in adults as well as neonates (Han et al., 2008; Neufeld and Zhao, 1995). Digit tip regeneration shares characteristics with amphibian limb regeneration in that both processes involve the formation of a blastema of undifferentiated, proliferating cells that express developmentally relevant genes;

however, the blastemas and the regeneration process itself are not equivalent (Han et al., 2008). One significant difference is that the mouse digit differentiates during regeneration by direct intramembranous ossification, whereas the digit tip differentiates during development by endochondral ossification. This deviation from the recapitulation of development (redevelopment) that is typical of amphibian limb regeneration (Bryant et al., 2002), suggests that digit tip regeneration has secondarily evolved from a non-regenerating condition (evolved regeneration), rather than a characteristic maintained from a regeneration-competent ancestor (Muneoka et al., 2008).

The level-dependent regeneration response of the mouse digit lends itself both to the discovery of requirements important for regeneration that can be tested in inhibition studies (loss of function), and to the design of regeneration therapies that can be tested on proximal amputation injuries (gain-of-function). In this study, we used the level-dependent regeneration response of the neonatal digit to explore the role of BMP signaling in digit regeneration. We report that BMP signaling is essential for the endogenous regeneration response and that proximal digit regeneration can be induced by treatment with either BMP2 or BMP7, but not BMP4. We also show that the induced proximal response involves the formation of a digit blastema and that redifferentiation occurs by endochondral, rather than direct, ossification. These findings indicate that cells at a non-regenerating amputation wound in a mammal have regenerative potential, and that the BMP signaling pathway distinguishes a wound healing event from a regenerative response.

MATERIALS AND METHODS

Mice and digit amputation

Mice (CD1) used in these studies were purchased from Charles River Laboratories (Wilmington, MA, USA) and Harlan Laboratories (Indianapolis, IN, USA). Experimental studies were carried out on digits 2 and 4 of both hindlimbs. Distal or proximal digit amputations were carried out as previously described (Han et al., 2008) at postnatal day 3 (PN3). Procedures for care and use of mice for this study were in compliance with Standard Operating Procedures approved by the Institutional Animal Care & Use Committee of Tulane University Health Science Center. For growth factor treatment, we used Affi-Gel Blue Gel beads (Bio-Rad, Hercules, CA, USA) as a microcarrier for delivery to the amputation wound. Beads (150 μm in diameter) were washed with PBS containing 0.1% BSA then soaked with recombinant human BMP2, 4 or 7, or recombinant mouse noggin (R&D Systems, Minneapolis, MN, USA) at a concentration of 0.5 mg/ml for 2 hours at room temperature. Control beads were soaked in PBS containing 0.1% BSA. Bead implantation was carried out 4 days post-amputation (DPA). Affi-Gel Blue Gel beads were pinned with a tungsten needle and briefly air dried, then inserted into the amputation wound between the wound epidermis and the amputated phalanx (Fig. 2A) and allowed to hydrate in situ before removing the needle. We used enhanced chondrogenesis in micromass cultures of E14 digit tip cells as a positive control for BMP signaling (X. Yang, unpublished).

Histological examination

To observe the skeletal pattern in wholemount, we stained digits with Alizarin Red S as previously described (Han et al., 2008). To quantify bone regeneration, we measured the proximal-distal length of each terminal phalanx (between 27-30 digits for each group). The experimental results were expressed as the mean ± one standard error. To determine newly forming bone, calcein was injected (10 mg/kg body weight, IP) at 14 or 35 days post-bead implantation (DPI) and analyzed 1 day later by fluorescence microscopy (Suzuki and Mathews, 1966). For histological analysis, digits were fixed with 4% paraformaldehyde in PBS at 4°C overnight, processed for standard paraffin histology, sectioned and stained with Mallory triple stain (Humason, 1962). In some cases, digits that were stained with Alizarin Red S for wholemount analysis were subsequently processed for paraffin histology after post-fixing in Z-FIX (Anatech LTD) and treatment with Decalcifier II (Surgipath). These digit samples had damaged epidermal tissues from processing; however, skeletal tissues were intact. Cell proliferation was examined by incorporation of BrdU (10 mM; 20 μl/g, IP), analyzing sections of digit samples using a BrdU Detection Kit II (Roche) according to manufacturer's instructions. To quantify proliferation, we counted BrdU-positive cells within defined fields (10,240 μm^2) of stump and connective tissue in representative sections of 5 digits for each treatment (BSA versus BMP7) and timepoint (3 and 7 DPI). Student's *t*-test was used to analyze for significance and error bars represent the standard deviation.

In situ hybridization and RT-PCR

Section in situ hybridization was performed to examine gene expression during digit tip regeneration, as described previously (Han et al., 2008). Antisense riboprobes were generated by in vitro transcription labeling with digoxigenin-UTP according to the manufacturer's instructions (Roche). The following cDNA fragments were used to generate antisense riboprobes: *Bmp2*, *Bmp7*, *Bmpr1a*, *Bmpr1b*, type II collagen (*Col2a1*), *Ihh*, type X collagen (*Col10a1*), *Msx1*, *Pedf* (*Serpinf1* – Mouse Genome Informatics), *Runx2*, *Dlx5*, *Sfrp2* and osteocalcin.

The expression of *Bmp2*, *Bmp7*, *Bmpr1a*, *Bmpr1b*, *Bmpr2* and *GAPDH* in the blastema was analyzed using semi-quantitative RT-PCR. Blastema tissue was collected from regenerating digit tips at 7 DPA and mesenchymal tissue of unamputated digit tip (PN10) was used for control. Total RNAs were isolated from both tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Primers used for RT-PCR are as follows: Bmp2-F 5′-GTTCCCTACAGGGAGAAC ACC-3′, Bmp2-R 5′-GCCTGCACAGATCTAGC-3′, Bmp7-F 5′-TCCAGGGAAAGCATAATTCG-3′, Bmp7-R 5′-ACCTCTCGTTGTCAAATCGC-3′, Bmpr1a-F 5′-TCGTCGTTGTATTACAGGAG-3′, Bmpr1a-R 5′-TTACATCCTGGGATTCAACC-3′, Bmpr1b-F 5′-GCTTTGGACTCATCCTCTGG, Bmpr1b-R 5′-CACTGGGCAGTAGGCTAACG, Bmpr2-F 5′-GGTAGATAGGAGGGAACGGC-3′, Bmpr2-R 5′-CACTGCCATTGTTGTTGACC-3′, GAPDH-F 5′-TTCCAGTATGATTCCACTCA-3′ and GAPDH-R 5′-CTGTAGCCATATTCATTGTC-3′.

RESULTS

Digit tip regeneration in neonatal mice is restricted to the distal half of the terminal phalangeal element (P3), whereas regenerative failure occurs following amputation through the proximal third of P3 (Han et al., 2008). Detailed histological analyses of wound healing following amputation showed considerable variability in the time for complete wound closure of the regenerating digit tip but less variability in the closure time of non-regenerating amputation wounds (Table 1). Wound closure in proximal P3 amputations or amputation at the P2 level was largely complete within 5 days; however, only 16.7% of the distal P3 amputations were closed by this time. The majority of distal P3 amputations were healed by 6 DPA (6/9); however, we did not observe 100% wound closure until 9 DPA. The variability in the rate of wound closure in distal amputations

Table 1. Wound closure after digit amputation

	P3		
	Distal	Proximal	P2
3 DPA	0% (0/12)	16.7% (2/12)	0% (0/6)
4 DPA	0% (0/10)	90.0% (9/10)	83.3% (5/6)
5 DPA	16.7% (2/12)	92.3% (12/13)	100% (4/4)
6 DPA	66.7% (6/9)	100% (7/7)	N/A
7 DPA	75.0% (6/8)	100% (8/8)	100% (3/3)
8 DPA	80.0% (12/15)	100% (15/15)	100% (3/3)
9 DPA	100% (6/6)	100% (6/6)	100% (3/3)

N/A, data not available; P3, third phalangeal element; P2, second phalangeal element.

compared with proximal amputations is curious as both wounds contain identical tissues, including nails, and the actual wound area of distal amputations is smaller than proximal amputations.

Previous studies on amputated fetal and neonatal digit tips implicated the BMP signaling pathway as a crucial regulator of regeneration. To investigate whether BMP signaling is required for mouse digit tip regeneration we treated distally amputated digit tips with the BMP antagonist noggin. Digits were distally amputated at postnatal day 3 (PN3) and, after a 4-day healing period, a single microcarrier bead carrying purified noggin was implanted between the wound epidermis and the amputated terminal phalangeal element. Digits were analyzed by wholemount skeletal staining for an anatomical regenerative response at 14 DPI. Control amputations, which received a bead carrying BSA, displayed a regenerative response comparable with the endogenous response (Fig. 1A), indicating that the implantation procedure did not interfere with the regeneration response. By contrast, delivering noggin to the amputation wound completely suppressed the regenerative response and resulted in truncated digit tips (Fig. 1B). We also carried out studies in which the endogenous regeneration response was supplemented by treatment with purified BMP4 introduced by bead implantation, and we found that excessive BMP4 did not modify proximal-distal digit outgrowth (Fig. 1C). We found similar results when beads containing BMP2 or BMP7 were implanted. These studies provide evidence that BMP signaling is required for digit tip regeneration and that excessive amounts of BMPs do not modify proximal-distal outgrowth during regeneration.

As noggin inhibits multiple BMPs and we had previously shown that *Bmp4* is expressed in the regeneration blastema (Han et al., 2008), we investigated the expression profiles of *Bmp2* and *Bmp7* and the BMP receptors *Bmpr1a*, *Bmpr1b* and *Bmpr2* during digit tip regeneration. Based on RT-PCR analysis, we found evidence that *Bmp2* and *Bmp7* transcripts were upregulated in digit blastema cells and that transcripts for the BMP receptors were present in cells both from the unamputated digit and the regeneration blastema (Fig. 1H). Using in situ hybridization, we found that *Bmp7* transcripts were localized to the nail epithelium, the proximal growth plate and the marrow region of the amputated bone stump (Fig. 1D). *Bmp2* transcripts were similarly distributed but, in addition, we found *Bmp2* expression in cells at the base of the blastema that interfaces with the amputated stump (Fig. 1E). Like *Bmp7*, transcripts for *Bmpr1a* localized to the nail epithelium, the distal epidermis, the proximal growth plate and the marrow region of the stump (Fig. 1F). *Bmpr1b* was also expressed in the distal epidermis, the proximal growth plate and in cells within the blastema (Fig. 1G). In summary, during digit tip regeneration, *Bmp7* and *Bmp2* are expressed in the marrow region of the stump, *Bmp2* is expressed in cells at the base of the blastema and *Bmp4* is expressed in the distal region of the blastema (Han et al., 2008). *Bmpr1a* and *Bmpr1b* are both expressed in the distal epidermis, whereas *Bmpr1a* is expressed in the stump marrow and *Bmpr1b* is expressed in cells of the blastema. These results demonstrate that multiple Bmp genes and their receptors are expressed in a region-specific manner during digit tip regeneration, and that BMP signaling is required for a successful regenerative response.

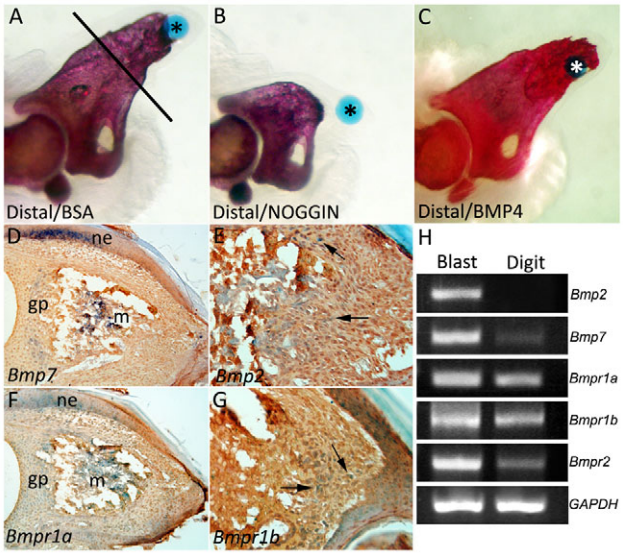

Fig. 1. BMP signaling and endogenous digit tip regeneration. (**A-C**) Wholemount stain with Alizarin Red 14 days after BSA (A), noggin (B) or BMP4 (C) bead (asterisk) implantation following amputation at a distal level (solid line in A). (**D-G**) In situ hybridization of *Bmp7* (D), *Bmp2* (E), *Bmpr1a* (F) and *Bmpr1b* (G) 7 days after distal amputation. *Bmp2*- and *Bmpr1b*-expressing cells in the blastema are indicated (arrows). ne, nail epithelium; gp, growth plate; m, bone marrow. (**H**) RT-PCR comparison of expression of *Bmp2*, *Bmp7*, *Bmpr1a*, *Bmpr1b* and *Bmpr2* in the blastema (Blast) and unamputated control tissue (Digit).

BMP7 and BMP2 induce digit regeneration from non-regenerating proximal amputation

The requirement of BMP signaling for digit regeneration raises the possibility that BMP signaling could be responsible for the failed regenerative response associated with proximal P3 amputation. The terminal phalanx forms by endochondral ossification and postnatal growth of this skeletal element involves a proximal epiphyseal growth plate (Han et al., 2008). Proximal amputation transects the phalangeal element just distal to the forming epiphyseal plate (see Fig. S1A in the supplementary material) and by 4 DPA, wound closure is largely completed (Table 1; see Fig. S1B in the supplementary material). In control proximal amputations, the skeletal stump undergoes some elongation from the proximal growth plate but its distal amputated surface ossifies and there is no evidence of a distal ossification center that typifies the normal digit tip (see Fig. S1C in the supplementary material). To test for an enhanced regenerative response, a single BMP-containing microcarrier bead was implanted between the wound epidermis and the skeletal stump after wound closure (Fig. 2A) and digits were analyzed at 14 DPI for skeletal outgrowth. Control digit amputations receiving a single BSA-containing bead failed to elicit a significant regeneration response and formed digits that were truncated at the proximal amputation level (Fig. 2B). Histological analyses and calcein labeling of BSA-treated proximal digit amputations indicated that distal elongation of the stump was confined to the formation of a bony cap that covered the stump bone (Fig. 2C,D).

Proximal P3 amputations treated with a single BMP bead can induce a regenerative response. Beads treated with purified BMP7 (Fig. 2E) or BMP2 (Fig. 2F) caused significant elongation of the terminal phalangeal element when analyzed at 14 DPI, whereas beads treated with BMP4 were unable to stimulate a statistically significant response (Fig. 3). The absence of a BMP4 response is curious; however, it is consistent with studies indicating that although BMP2 and BMP7 are osteogenic in vivo, ectopic expression of BMP4 failed to elicit a similar response (Kang et al., 2004). At 14 DPI, the bulk of the regenerated digit tip is composed of newly formed trabecular bone that is histologically distinct but

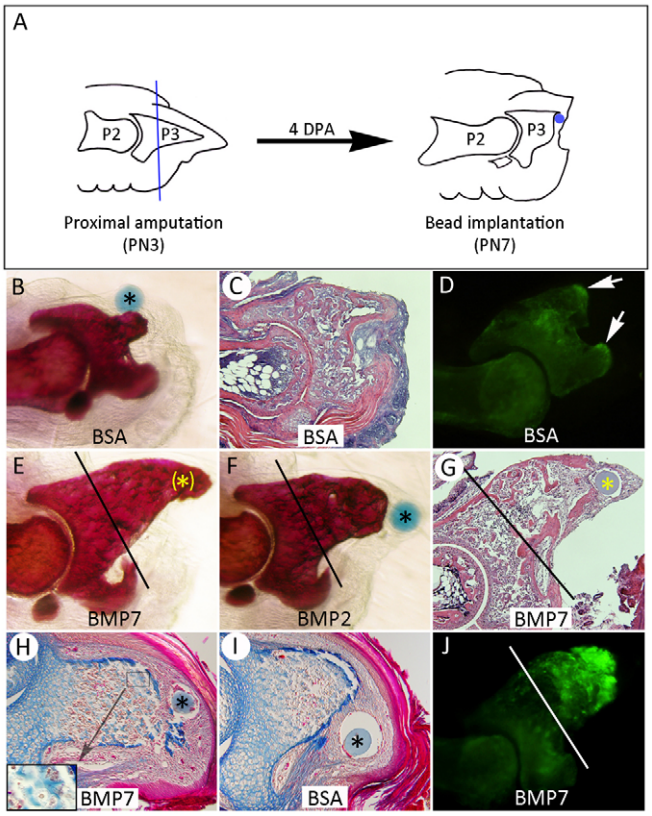

Fig. 2. BMP induces bone regeneration from the proximally amputated digit tip. (**A**) Diagram showing the level of proximal amputation and the position of bead placement. (**B-D**,**I**) Control digits with BSA bead. (**E-H**,**J**) Experimental digits with BMP bead. (B,E,F) Wholemount Alizarin Red-stained samples 14 DPI treated with BSA (B), BMP7 (E) or BMP2 (F). (C,G) Wholemount stained samples processed for the histological analysis using Mallory triple stain. (D,J) 14 DPI digits stained with calcein to identify regions of ossification. (D) BSA-treated control digit showing the formation of ossification caps (white arrows). (J) BMP7 bead-implanted digit showing a robust distal ossification center. (H,I) 7 DPI samples processed for histological staining with Mallory triple stain. (H) In BMP7-treated digits, chondrocytes (inset) are observed in the regenerate newly forming bone. (I) BSA control digits do not form chondrocytes in the distal stump. Solid line indicates the amputation plane, asterisk indicates implanted bead.

integrated with the stump bone to reform the terminal phalanx (Fig. 2G). Histological analysis at 7 DPI indicates the presence of chondrogenic cells in the distal region of the BMP7-treated stump and their absence in stage-matched control digits (Fig. 2H,I). Calcein labeling studies at 14 DPI demonstrated intense staining throughout the regenerated digit tip (Fig. 2J). We note that the proximal amputation level is associated with a hole in the ventrolateral part of the P3 bone (Fig. 1A) that connects the marrow region with the lateral dermis. This digital os hole does not completely reform in BMP-induced regenerates and serves as an additional anatomical marker for the level of amputation, thus eliminating any doubts that the regeneration response might result from variability of amputation level. Using the proximal-distal length of the terminal phalanx at 14 DPI as a way to quantify this response, our measurements demonstrate an induction of skeletal elongation of 45% and 58% (as compared with control digits treated

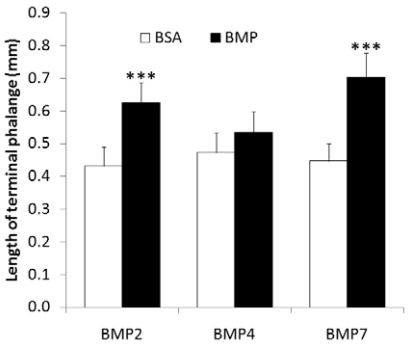

Fig. 3. Comparison of the proximal-distal length of the terminal phalangeal bone at 14 DPI in BSA- and BMP-treated digits. Student's *t*-test ± s.e.m. ***, $P< 0.001$.

with BSA beads) for BMP2 and BMP7, respectively (Fig. 3). These data indicate that the amputated neonatal digit can be stimulated to regenerate by a single treatment with either BMP2 or BMP7.

As BMP7 provided the highest level of skeletal elongation at 14 DPI, we focused our studies on this response. We next analyzed digits at 35 DPI to compare the final anatomy of the response with that of control BSA-treated and unamputated digits. The external anatomy of the 5 week regenerates was generally similar to that of unamputated digits and dramatically different from truncated BSA-treated controls (Fig. 4A-C). The nail that surrounds the terminal phalanx appears normal, although it is generally blunted at the tip. The terminal phalanx is variable in length, with some samples approaching the proximal-distal length of control unamputated digits, and all samples displaying a regenerative response by comparison with the proximally truncated BSA control digits (Fig. 4D,E). The distal tip of the regenerated digit is generally rounded with only the occasional sample forming a pointed tip characteristic of unamputated digits (Fig. 4F). Calcein incorporation studies indicate extensive new bone deposition even at 35 DPI (Fig. 4F) suggesting that BMP7 establishes an ossification center that is able to continue after the treatment is exhausted.

Blastema formation in BMP7-induced regeneration

Because we are able to induce a long-term regeneration response with a single application of BMP7, we hypothesize that BMP7 is acting to induce a morphogenetic center (such as a blastema) that subsequently organizes the regeneration response. To explore this possibility, we began by documenting changes in cell proliferation following amputation injury to determine whether there is evidence of blastema formation. During endogenous digit tip regeneration, a digit blastema containing proliferating cells is present by 7 days (Han et al., 2008). Simple proximal amputation results in wound closure by 4 days (Table 1), at which time the wound epidermis directly abuts the amputated skeletal stump, and few proliferating cells are observed (Fig. 5A). At 7 DPA, there is a thin layer of connective tissue that invades the space between the skeletal stump and the wound epidermis (Fig. 5B). In proximal amputations that received an implanted BSA control microcarrier bead, we see a distal accumulation of cells by 3 DPI but there is no increase in proliferation (Fig. 5C). By 7 DPI, there are few proliferating cells (Fig. 5D) and the stump has differentiated a periosteum across the amputated surface (Fig. 7D). These observations suggest that bead

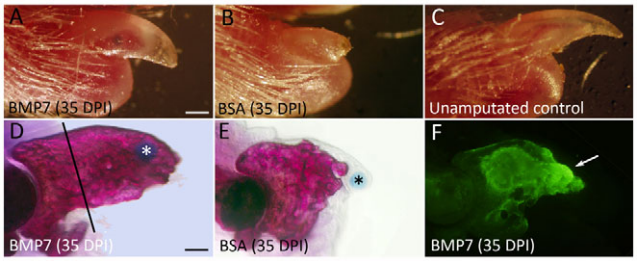

Fig. 4. Final anatomy of regenerated digits at 35 DPI. (**A-C**) Final anatomy after BMP7 (A) and BSA (B) treatment compared with a stage-matched unamputated digit (C). A near-normal digit tip is regenerated following BMP7 treatment. (**D**,**E**) Wholemount bone stain (Alizarin Red S) of BMP7- (D) or BSA (E)-treated digits. (**F**) Calcein labeling of the BMP7-treated digit at 35 DPI showing the persistence of a distal ossification center (arrow). Solid line indicates the amputation plane, asterisk indicates implanted bead. Scale bars: 200 μm.

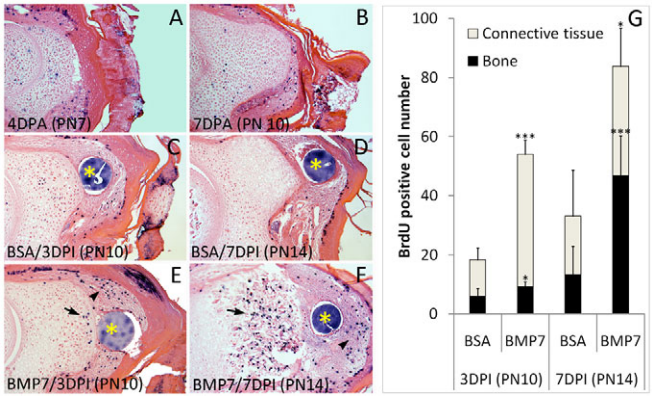

Fig. 5. Cell proliferation during BMP7-induced regeneration. (**A-F**) BrdU incorporation at 4 (A) and 7 (B) DPA (PN7 and PN10, respectively) after proximal amputation. BrdU incorporation at 3 (C) and 7 (D) DPI (PN10 and PN14, respectively) after treatment with BSA. BrdU incorporation at 3 (E) and 7 (F) DPI (PN10 and PN14, respectively) after treatment with BMP7. Increased zones of proliferation are induced by BMP7 in the connective tissue (arrowheads) and in the bone stump (arrows). Asterisks indicates implanted beads. (**G**) BrdU labeling was quantified by cell counts in comparable regions of BSA and BMP7-treated digits at 3 and 7 DPI. The data show BMP7-enhanced proliferation in the connective tissue at 3 DPI, and in both connective tissue and the stump at 7 DPI. Student's *t*-test ± s.d.; *, $P<0.05$, ***, $P<0.001$.

implantation itself is sufficient to induce the accumulation of cells at the amputation wound but that the conditions are insufficient to induce a growth response.

In digits that received a BMP7 bead, a digit blastema containing proliferating cells forms at the amputation wound by 3 DPI. Enhanced cell proliferation of connective tissue cells surrounding the bead, and cells of the skeletal stump just proximal to the bead, is observed (Fig. 5E). By 7 DPI, there are two distinct proliferation zones that can be identified by BrdU incorporation: (1) a distal zone between the BMP7 bead and the wound epidermis and (2) within the stump just proximal to the BMP7 bead (Fig. 5F). To quantify this proliferative effect, we counted BrdU-positive cells within the connective tissue surrounding the bead and within the distal stump just proximal to the bead, comparing BMP7-treated digits with control digits at 3 and 7 DPI (Fig. 5G). The data indicate that BMP7 treatment results in enhanced proliferation of connective tissue cells, as well as stump cells, at both timepoints analyzed.

Another aspect of mammalian digit blastema formation during regeneration is the re-expression of relevant developmental genes (Gardiner, 2005). *Msx1* is expressed at the embryonic digit tip and has been found to be required for embryonic digit tip regeneration (Han et al., 2003). Postnatally, *Msx1* is expressed in the dorsal dermis subjacent to the nail matrix (Reginelli et al., 1995) (see Fig. S2A in the supplementary material), and it is transiently upregulated during digit blastema formation in neonatal digit tip regeneration (Han et al., 2008). Similarly, in BMP7-induced proximal digit regeneration, we observe that *Msx1* is transiently upregulated in the dorsal region of the induced digit blastema at 3 DPI (Fig. 6B) but its expression is absent in the digit blastema at 7 DPI (Fig. 6D). In control BSA-treated amputations, *Msx1* expression is restricted to the proximal-dorsal dermis at both 3 and 7 DPI (Fig. 6A,C). The WNT antagonist *Sfrp2* is a gene that is prominently expressed in the dorsal dermis of the neonatal digit (see Fig. S2B in the supplementary material); however, unlike *Msx1*, the *Sfrp2* expression domain is not modified during digit blastema formation associated with endogenous digit tip regeneration (see Fig. S2C in the supplementary material). In BMP7-induced regeneration, we find *Sfrp2*-expressing cells associated with the BMP7 bead at 3 DPI (Fig. 6F) but by 7DPI, there are no *Sfrp2*-expressing cells present in the digit blastema (Fig. 6H). In control BSA-treated amputations, *Sfrp2* expression is restricted to the proximal-dorsal dermis at both 3 and 7 DPI (Fig. 6E,G). *Msx2* is normally expressed in the nail organ of the neonatal digit and is transiently upregulated in the dermis during digit tip regeneration (Han et al., 2008); however, it is not induced in the digit blastema at either 3 or 7 DPI by BMP7 (data not shown).

During digit tip formation, *Pedf* is first expressed in cells within the forming bone marrow (see Fig. S2D in the supplementary material). During endogenous digit tip regeneration, *Pedf* is also expressed in the early digit blastema in regions subjacent to the wound epidermis (Muneoka et al., 2008). In proximal digit tip amputations treated with BMP7 beads, we find *Pedf* expression is upregulated in cells of both the stump and digit blastema at both 3 and 7 DPI (Fig. 6J,L). *Pedf* is not upregulated in control digit amputations treated with BSA at 3 or 7 DPI (Fig. 6I,K). The normally restricted expression of *Pedf* in the digit bone marrow, along with the continuity of *Pedf*-expressing cells between the bone marrow and the digit blastema, suggest that the digit blastema is derived in part from bone marrow cells. We note that there is variable expression of *Msx1*, *Sfrp2* and *Pedf* in the nail epidermis, which appears to be linked to injury rather than BMP7 treatment.

BMP7 induces endochondral ossification

The terminal phalanx of the mouse forms by endochondral ossification and it elongates by the combined growth of the proximally located epiphyseal growth plate, and by appositional ossification that occurs at the digit tip (Han et al., 2008; Muneoka et al., 2008). At birth, the terminal phalangeal element is triangular-shaped with proliferating chondrocytes expressing type II collagen (*Col2a1*) at its proximal base, prehypertrophic chondrocytes expressing *Ihh* at an intermediate zone and hypertrophic chondrocytes expressing type X collagen (*Col10a1*) at the distal apex. Osteocalcin-expressing osteoblasts are first identified at the distal apex surrounding the hypertrophic chondrocytes. As the digit tip forms, the three chondrocytic zones become compressed

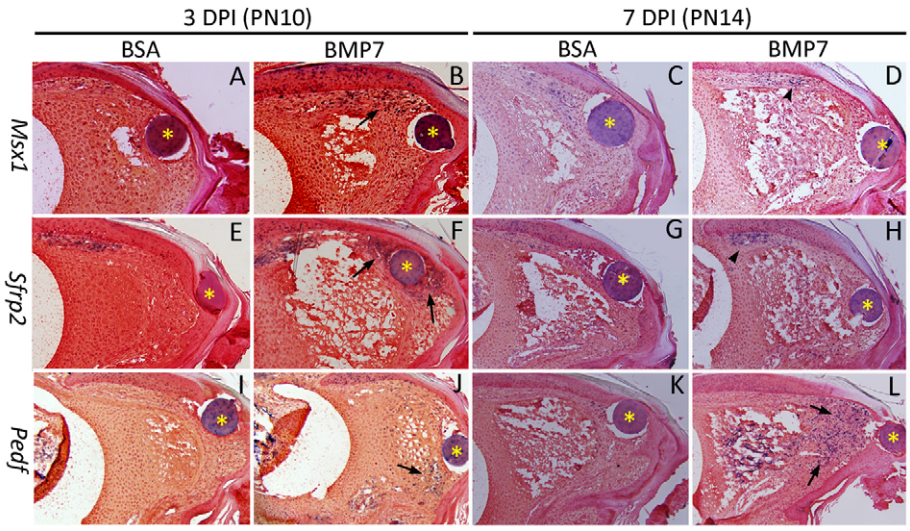

Fig. 6. Gene expression in the digit blastema. (**A-L**) In situ hybridization of *Msx1* (A-D), *Sfrp2* (E-H) and *Pedf* (I-L) in BMP7-induced regenerates and BSA controls at 3 and 7 DPI. *Msx1* (B) and *Sfrp2* (F) show BMP7-induced upregulation (arrows) at 3 DPI as compared with stage-matched controls (A,E). At 7 DPI, *Msx1* (D) and *Sfrp2* (H) expression is absent from the blastema and is restricted to the dorsal-proximal mesenchyme (arrowheads), similar to in control digits (C,G). Cells expressing *Pedf* are enhanced at 3 DPI in BMP7-treated digits (J, arrow). At 7 DPI, *Pedf* expression is prominent in the BMP7-induced digit blastema and in the marrow region (L, arrows), but largely absent in the stage-matched BSA controls (K). Asterisks indicate implanted beads.

proximally to form the proximal epiphyseal growth plate of the digit. The regeneration of the digit tip that occurs endogenously does not recapitulate these developmental events but, instead, involves digit blastema formation and direct ossification by a process that appears most similar to intramembranous ossification (Han et al., 2008). Because of this, we have proposed that digit tip regeneration in the mouse is a response that evolved secondarily from a non-regenerative digit tip, utilizing developmental mechanisms that are novel for digit tip development (Muneoka et al., 2008). With this in mind, we have investigated the mechanism of skeletal differentiation that is triggered by BMP7 during induced regeneration to determine whether skeletal regrowth occurred by endochondral ossification (redevelopment) or by direct ossification (evolved regeneration).

We characterized ossification by analyzing the expression of endochondral (*Col2a1*, *Ihh*, *Col10a1*) and osteogenic (osteocalcin, *Dlx5*, *Runx2*) marker genes by in situ hybridization. Our studies focused at 7 DPI because at this stage we find histological evidence of chondrogenesis (Fig. 2H) associated with regions of the stump with enhanced proliferation (Fig. 5G). In control BSA-treated amputated digits, we found no change in expression of any of the endochondral marker genes (Fig. 5A-C). In all cases, the expression domains were localized to the proximal base of the terminal phalangeal element and were indistinguishable from unamputated digit tips of a similar age. Similarly, the expression of *Dlx5* (see Fig. S3A in the supplementary material), *Runx2* (see Fig. S3C in the supplementary material) and osteocalcin (Fig. 7D) in BSA-treated amputations was largely similar to unamputated digits, with the only variation being the formation of an ossification cap across the amputated stump.

In BMP7-induced regenerates at 7 DPI, we found expression of osteogenic genes *Dlx5* (see Fig. S3B in the supplementary material), *Runx2* (see Fig. S3D in the supplementary material) and osteocalcin (Fig. 7H) throughout the regenerating skeletal stump, consistent with the enhanced osteogenesis indicated by calcein incorporation (Fig. 2J). To establish whether the regenerated bone formed by direct ossification versus endochondral ossification, we examined the expression of endochondral marker genes. We found ectopic expression domains of all three endochondral markers, *Co2a1*, *Ihh* and *Col10a1*, within in the regenerating region of the skeletal stump undergoing ossification (Fig. 7E-G). Importantly, these ectopic domains were organized proximal-distally, in a manner identical to their pattern in the developing P3 element, i.e. the *Col10a1* domain is localized at the apex of the regenerating stump, the *Ihh* domain is localized just proximal to *Col10a1* and the *Col2a1* domain is proximal to *Ihh*. This organization of the ectopic endochondral marker domains is suggestive of a newly formed endochondral ossification center associated with the induced regenerative response. The formation of an ectopic endochondral growth zone provides a plausible explanation for how a single treatment with BMP7 can initiate a response that continues for several weeks after treatment and results in the restoration of the terminal phalanx. These observations also provide evidence that BMP7 stimulates regeneration by triggering the activation of a developmental program used initially to form the digit tip during embryogenesis and not the endogenous regenerative program.

DISCUSSION

The induction of a regeneration response at a non-regenerating mammalian wound is arguably one of the biggest challenges in regeneration biology. We have used a relatively simple model involving the level-dependent regeneration response of the mouse digit tip to test the involvement of the BMP signaling pathway in an endogenous regeneration and also in a failed regenerative response. Based on loss-of-function studies, we show that endogenous regeneration is inhibited when the amputation wound is treated with a BMP-specific antagonist and conclude that BMP signaling is required for digit tip regeneration. In gain-of-function studies, we are able to induce regeneration in amputation wounds that are regeneration-incompetent by providing a source of either BMP7 or BMP2, thus showing that the absence of a BMP source is responsible for regenerative failure in this model. Finally, we show that the induced regeneration response represents a case in which cells undergo a redevelopment response rather than the evolved regeneration response that typifies endogenous regeneration. Overall, these studies provide the first clear demonstration in a mammal that a regeneration-incompetent amputation wound can be transitioned into a regeneration-competent wound by treatment with a single growth factor.

Digit tip regeneration can be divided into three distinct phases: wound healing, digit blastema formation and redifferentiation (Muneoka et al., 2008). Although the wound healing phase has not been extensively studied, it seems probable that the amputation

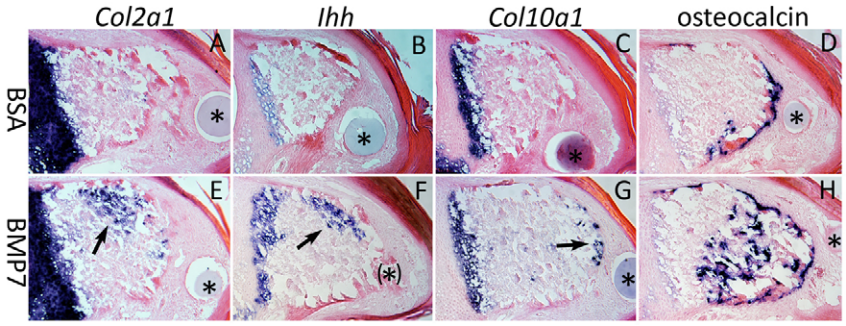

Fig. 7. Endochondral marker genes are induced by BMP7 treatment. (**A-H**) At 7 DPI, the expression of endochondral marker genes (*Col2a1*, *Ihh* and *Col10a1*) and osteogenic marker genes (osteocalcin) was examined by in situ hybridization in control (A-D) and BMP7-induced regenerates (E-H). BSA control digits display expression of endochondral marker genes at the proximal base of the digit (A-C), with osteocalcin expression capping the distal digit stump (D). In BMP7-treated digits, ectopic expression domains of the endochondral marker genes (arrows in E-G) are induced in the regenerate, and enhanced osteocalcin expression is observed throughout the distal stump (H). Asterisks indicate implanted beads.

healing response largely parallels that of full thickness wounds. The early events in the healing of full thickness skin wounds includes the accumulation of platelets that are involved in the formation of the fibrin clot, and a subsequent inflammation response that brings neutrophils and monocytes to the wound site. These early responses modify the wound site by the production of an array of cytokines, growth factors and bactericidal peptides and set the stage for the structural repair of the wound (Stocum, 2006). Like skin wounds, re-epithelialization of digit amputation wounds occurs very slowly, and we note that regenerating wounds close at a slower and more variable rate than non-regenerating wounds. A similar relationship between the quality of the healing response and a slower rate of wound closure has been reported in mice over-expressing the activin antagonist gene follistatin (Wankell et al., 2001). Thus, the evidence suggests that, in mammals, slow wound closure is associated with an enhanced regenerative response, whereas rapid wound closure is associated with regenerative failure. As we are able to induce a regenerative response with BMP7 after wound closure is completed, our studies demonstrate that all of the early events associated with the wound healing process, including wound closure and inflammation, do not irreversibly antagonize the regenerative potential of the cells at the wound site.

The mammalian blastema is defined as an aggregate of proliferating undifferentiated cells involved in the regenerative response (Muneoka et al., 2008). The combined results from our histological, proliferation and gene expression studies indicate that a digit blastema forms during a BMP7-induced response, whereas a blastema fails to form in BSA-treated digits. The proliferative response is similar to that documented during endogenous digit tip regeneration in that two distinct proliferative zones form, one associated with the connective tissue and one associated with the distal skeletal stump (Han et al., 2008). In BMP7-induced regeneration we find that enhanced proliferation is initially associated with the bead, but later appears to be bead-independent. It is generally accepted that growth factor release from agarose beads is exhausted within days after implantation (Fallon et al., 1994; L. Marrero, unpublished), thus the existence of regenerative growth zones at later stages indicates that BMP7 initiates a self-sustaining regenerative growth response. How BMP signaling facilitates this response is unclear; however, we note that some developmental genes (*Msx1*, *Srfp2*) are transiently expressed during blastema formation. The transient upregulation of the homeobox-containing transcriptional repressor, *Msx1*, is of interest because *Msx1* has known functions in controlling proliferation and differentiation (Hu et al., 2001; Odelberg et al., 2000) and is required for embryonic digit regeneration (Han et al., 2003). Conversely, digit blastemal cells express *Pedf*, a gene not linked to digit development but expressed postnatally by resident cells in the bone marrow and a potent anti-angiogenic factor (Filleur et al., 2009). Pedf has also been identified as a chemoattractant for fibroblasts (Sarojini et al., 2008), which raises the possibility for a role in cell recruitment during the regenerative response.

Redifferentiation of the BMP7-induced regenerates demonstrates that the induced response is distinct from endogenous regeneration and that it involves a redevelopment response. During digit formation, ossification of the terminal phalanx initiates at the digit tip with the differentiation of osteoblasts (expressing osteocalcin) surrounding an apical population of hypertrophic chondrocytes (expressing *Col10a1*). The proximal half of the terminal phalanx consists of proliferating chondrocytes (expressing *Col2a1*), and between the proliferating chondrocytes and the hypertrophic chondrocytes, there is a layer of pre-hypertrophic chondrocytes that express *Ihh*. (Han et al., 2008). Postnatally, the endochondral marker genes are expressed in domains within the epiphyseal growth plate at the base of the terminal phalanx. Endogenous digit tip regeneration occurs by direct ossification and the endochondral genes are not re-expressed in the regenerates (Han et al., 2008). By contrast, BMP7-induced regeneration is associated with the re-expression of endochondral marker genes, and their expression domains follow the proximal-distal pattern of the developing terminal phalanx. These results clearly show that the BMP7-induced response is distinct from endogenous regeneration and that it involves modification of the amputation wound in a way that allows cells to reactivate differentiation programs that restore the amputated structure. The reactivation of developmental programs effectively represents a reprogramming event that has obvious parallels with the process of dedifferentiation in limb regeneration (Brockes and Kumar, 2005; Gardiner, 2005; Han et al., 2005). Overall, the finding that developmental programs can be reactivated at a postnatal mammalian injury site provides some promise that similar reprogramming events might be inducible in adult tissues.

How BMP7 treatment induces this regenerative response is not clear at this time. BMP signaling is known to play crucial roles in both limb development (Robert, 2007) and skeletal repair following injury (Schindeler et al., 2008). The during early limb development, BMP signaling is implicated in the proper

formation of the apical ectodermal ridge (AER) (Ahn et al., 2001; Pizette et al., 2001), and establishing a wound epidermis that shares characteristics with the AER is a crucial early event in amphibian limb regeneration (Christensen and Tassava, 2000; Satoh et al., 2008). Thus, one possible mechanism involves a modification of the mammalian wound epidermis that functions to induce regenerative outgrowth. Alternatively, BMP signaling is essential for the differentiation of the limb skeleton (Bandyopadhyay et al., 2006) and our finding that ectopic endochondral ossification is induced by BMP7 is consistent with a role in inducing skeletogenesis. The onset of expression of the endochondral genes during regeneration, however, occurs after BMP release from the microcarrier bead is exhausted, thus a direct effect of BMP7 on endochondral ossification seems unlikely. BMP7 has been shown to induce ectopic bone formation when administered immediately after limb amputation in neonatal mice (Masaki and Ide, 2007). BMPs have long been known for their ability to induce ectopic bone when placed at a subcutaneous or intramuscular location (see Reddi, 1998); however, the morphology of the ectopic skeletal elements induced by BMP7 at neonatal limb amputations display a pattern that is linked to the amputation level (Masaki and Ide, 2007). In digit tip regeneration we also find evidence for a level-dependent response. Endogenous digit tip regeneration, which is BMP-dependent, occurs by direct ossification (Han et al., 2008), whereas BMP7-induced regeneration occurs by endochondral ossification. Thus it appears that, even within the confines of the terminal phalangeal element, digit cells can respond in a position-specific manner. The role of positional information in amphibian limb regeneration has long been recognized as an important aspect for a successful response and that connective tissue fibroblasts play a key role in blastema formation and patterning the regenerative response (Gardiner, 2005). There is evidence from microarray analyses of human cells that fibroblasts display transcriptomes that vary with position in the adult body (Chang et al., 2002; Rinn et al., 2006), suggesting that some measure of positional information is maintained even in non-regenerating adult tissues. Our results, combined with those of Masaki and Ide (Masaki and Ide, 2007), provide evidence for an interface between BMP signaling and the positional identity of cells at amputation wounds in modulating the mammalian injury response.

Acknowledgements

We thank Juhee Haam for sharing preliminary results and Akira Satoh for comments on the manuscript. Research funded by grants R01HD043277 and P01HD022610 from the NIH, W911NF-06-1-0161 from DARPA and the John L. and Mary Wright Ebaugh Endowment Fund at Tulane University. Deposited in PMC for immediate release.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.042424/-/DC1

References

Ahn, K., Mishina, Y., Hanks, M. C., Behringer, R. R. and Crenshaw, E. B., 3rd (2001). BMPR-IA signaling is required for the formation of the apical ectodermal ridge and dorsal-ventral patterning of the limb. *Development* **128**, 4449-4461.

Allan, C. H., Fleckman, P., Fernandes, R. J., Hager, B., James, J., Wisecarver, Z., Satterstrom, F. K., Gutierrez, A., Norman, A., Pirrone, A. et al. (2006). Tissue response and Msx1 expression after human fetal digit tip amputation in vitro. *Wound Repair Regen.* **14**, 398-404.

Bandyopadhyay, A., Tsuji, K., Cox, K., Harfe, B. D., Rosen, V. and Tabin, C. J. (2006). Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. *PLoS Genet.* **2**, e216.

Brockes, J. P. and Kumar, A. (2005). Appendage regeneration in adult vertebrates and implications for regenerative medicine. *Science* **310**, 1919-1923.

Bryant, S. V., Endo, T. and Gardiner, D. M. (2002). Vertebrate limb regeneration and the origin of limb stem cells. *Int. J. Dev. Biol.* **46**, 887-896.

Carlson, B. M. (2007). *Principles of Regenerative Biology*. Burlington, MA: Academic Press.

Chan, W. Y., Lee, K. K. and Tam, P. P. (1991). Regenerative capacity of forelimb buds after amputation in mouse embryos at the early-organogenesis stage. *J. Exp. Zool.* **260**, 74-83.

Chang, H. Y., Chi, J. T., Dudoit, S., Bondre, C., van de Rijn, M., Botstein, D. and Brown, P. O. (2002). Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proc. Natl. Acad. Sci. USA* **99**, 12877-12882.

Christensen, R. N. and Tassava, R. A. (2000). Apical epithelial cap morphology and fibronectin gene expression in regenerating axolotl limbs. *Dev. Dyn.* **217**, 216-224.

Deuchar, E. (1976). Regeneration of amputated limb-buds in early rat embryos. *J. Embryol. Exp. Morphol.* **35**, 345-354.

Fallon, J. F., Lopez, A., Ros, M. A., Savage, M. P., Olwin, B. B. and Simandl, B. K. (1994). FGF-2: apical ectodermal ridge growth signal for chick limb development. *Science* **264**, 104-107.

Filleur, S., Nelius, T., de Riese, W. and Kennedy, R. C. (2009). Characterization of PEDF: a multi-functional serpin family protein. *J. Cell. Biochem.* **106**, 769-775.

Fleming, M. W. and Tassava, R. A. (1981). Preamputation and postamputation histology of the neonatal opossum hindlimb: implications for regeneration experiments. *J. Exp. Zool.* **215**, 143-149.

Gardiner, D. M. (2005). Ontogenetic decline of regenerative ability and the stimulation of human regeneration. *Rejuvenation Res.* **8**, 141-153.

Han, M., Yang, X., Farrington, J. E. and Muneoka, K. (2003). Digit regeneration is regulated by Msx1 and BMP4 in fetal mice. *Development* **130**, 5123-5132.

Han, M., Yang, X., Taylor, G., Burdsal, C. A., Anderson, R. A. and Muneoka, K. (2005). Limb regeneration in higher vertebrates: developing a roadmap. *Anat. Rec. B New Anat.* **287**, 14-24.

Han, M., Yang, X., Lee, J., Allan, C. H. and Muneoka, K. (2008). Development and regeneration of the neonatal digit tip in mice. *Dev. Biol.* **315**, 125-135.

Hu, G., Lee, H., Price, S. M., Shen, M. M. and Abate-Shen, C. (2001). Msx homeobox genes inhibit differentiation through upregulation of cyclin D1. *Development* **128**, 2373-2384.

Humason, G. L. (1962). *Animal Tissue Techniques*. San Francisco: W. H. Freeman & Co.

Kang, Q., Sun, M. H., Cheng, H., Peng, Y., Montag, A. G., Deyrup, A. T., Jiang, W., Luu, H. H., Luo, J., Szatkowski, J. P. et al. (2004). Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery. *Gene Ther.* **11**, 1312-1320.

Kawakami, Y., Rodriguez Esteban, C., Raya, M., Kawakami, H., Marti, M., Dubova, I. and Izpisua Belmonte, J. C. (2006). Wnt/beta-catenin signaling regulates vertebrate limb regeneration. *Genes Dev.* **20**, 3232-3237.

Kostakopoulou, K., Vogel, A., Brickell, P. and Tickle, C. (1996). 'Regeneration' of wing bud stumps of chick embryos and reactivation of Msx-1 and Shh expression in response to FGF-4 and ridge signals. *Mech. Dev.* **55**, 119-131.

Masaki, H. and Ide, H. (2007). Regeneration potency of mouse limbs. *Dev. Growth Differ.* **49**, 89-98.

Mizell, M. (1968). Limb regeneration: induction in the newborn opossum. *Science* **161**, 283-286.

Muller, T. L., Ngo-Muller, V., Reginelli, A., Taylor, G., Anderson, R. and Muneoka, K. (1999). Regeneration in higher vertebrates: limb buds and digit tips. *Semin. Cell Dev. Biol.* **10**, 405-413.

Muneoka, K. and Sassoon, D. (1992). Molecular aspects of regeneration in developing vertebrate limbs. *Dev. Biol.* **152**, 37-49.

Muneoka, K., Allan, C. H., Yang, X., Lee, J. and Han, M. (2008). Mammalian regeneration and regenerative medicine. *Birth Defects Res. C Embryo Today* **84**, 265-280.

Neufeld, D. A. and Zhao, W. (1995). Bone regrowth after digit tip amputation in mice is equivalent in adults and neonates. *Wound Repair Regen.* **3**, 461-466.

Odelberg, S. J., Kollhoff, A. and Keating, M. T. (2000). Dedifferentiation of mammalian myotubes induced by msx1. *Cell* **103**, 1099-1109.

Pizette, S., Abate-Shen, C. and Niswander, L. (2001). BMP controls proximodistal outgrowth, via induction of the apical ectodermal ridge, and dorsoventral patterning in the vertebrate limb. *Development* **128**, 4463-4474.

Reddi, A. H. (1998). Role of morphogenetic proteins in skeletal tissue engineering and regeneration. *Nat. Biotechnol.* **16**, 247-252.

Reginelli, A. D., Wang, Y. Q., Sassoon, D. and Muneoka, K. (1995). Digit tip regeneration correlates with regions of Msx1 (Hox 7) expression in fetal and newborn mice. *Development* **121**, 1065-1076.

Rinn, J. L., Bondre, C., Gladstone, H. B., Brown, P. O. and Chang, H. Y. (2006). Anatomic demarcation by positional variation in fibroblast gene expression programs. *PLoS Genet.* **2**, e119.

Robert, B. (2007). Bone morphogenetic protein signaling in limb outgrowth and patterning. *Dev. Growth Differ.* **49**, 455-468.

Sarojini, H., Estrada, R., Lu, H., Dekova, S., Lee, M. J., Gray, R. D. and Wang, E. (2008). PEDF from mouse mesenchymal stem cell secretome attracts fibroblasts. *J. Cell. Biochem.* **104**, 1793-1802.
Satoh, A., Graham, G. M., Bryant, S. V. and Gardiner, D. M. (2008). Neurotrophic regulation of epidermal dedifferentiation during wound healing and limb regeneration in the axolotl (Ambystoma mexicanum). *Dev. Biol.* **319**, 321-335.
Schindeler, A., McDonald, M. M., Bokko, P. and Little, D. G. (2008). Bone remodeling during fracture repair: The cellular picture. *Semin. Cell Dev. Biol.* **19**, 459-466.
Singer, M. (1952). The influence of the nerve in regeneration of the amphibian extremity. *Q. Rev. Biol.* **27**, 169-200.
Stocum, D. L. (2006). *Regenerative Biology and Medicine*. Burlington, MA: Academic Press.
Suzuki, H. K. and Mathews, A. (1966). Two-color fluorescent labeling of mineralizing tissues with tetracycline and 2,4-bis[N,N'-di-(carbomethyl)aminomethyl] fluorescein. *Stain Technol.* **41**, 57-60.
Taylor, G. P., Anderson, R., Reginelli, A. D. and Muneoka, K. (1994). FGF-2 induces regeneration of the chick limb bud. *Dev. Biol.* **163**, 282-284.
Wanek, N., Muneoka, K. and Bryant, S. V. (1989). Evidence for regulation following amputation and tissue grafting in the developing mouse limb. *J. Exp. Zool.* **249**, 55-61.
Wankell, M., Munz, B., Hubner, G., Hans, W., Wolf, E., Goppelt, A. and Werner, S. (2001). Impaired wound healing in transgenic mice overexpressing the activin antagonist follistatin in the epidermis. *EMBO J.* **20**, 5361-5372.
Yakushiji, N., Yokoyama, H. and Tamura, K. (2009). Repatterning in amphibian limb regeneration: A model for study of genetic and epigenetic control of organ regeneration. *Semin. Cell Dev. Biol.* **20**, 565-574.